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ZP3-dependent Activation of Sperm Cation Channels Regulates Acrosomal Secretion During Mammalian Fertilization

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Abstract. The sperm acrosome reaction is a Ca^{2+}-dependent secretory event required for fertilization. Adhesion to the egg's zona pellucida promotes Ca^{2+} influx through voltage-sensitive channels, thereby initiating secretion. We used potentiometric fluorescent probes to determine the role of sperm membrane potential in regulating Ca^{2+} entry. ZP3, the glycoprotein agonist of the zona pellucida, depolarizes sperm membranes by activating a pertussis toxin–insensitive mechanism with the characteristics of a poorly selective cation channel. ZP3 also activates a pertussis toxin–sensitive pathway that produces a transient rise in internal pH. The concerted effects of depolarization and alkalinization open voltage-sensitive Ca^{2+} channels. These observations suggest that mammalian sperm utilize membrane potential–dependent signal transduction mechanisms and that a depolarization pathway is an upstream transducing element coupling adhesion to secretion during fertilization.

THE acrosome reaction is a Ca^{2+}-dependent secretory event in sperm that is an obligatory early step in the fertilization process. It results in the release of acrosomal granule contents, the extensive reorganization of sperm surface proteins, and the display of new membrane domains at the surface. Only sperm that have completed the acrosome reaction are capable of fusing with eggs (reviewed by 67).

In mammals, acrosome reactions are triggered by contact with the zona pellucida (ZP),[1] the egg's extracellular matrix, or by treatment with soluble ZP extracts (reviewed by 60). Such extracts contain three glycoproteins, designated ZP1, ZP2, and ZP3 (reviewed by 63 and 64). ZP3 completely accounts for the acrosome reaction–inducing agonist activity, as demonstrated by studies with highly purified oocyte ZP3 (10, 45) and with recombinant ZP3 expressed by mammalian cells (5, 35). The mechanism of ZP3 action is presently a central, unresolved aspect of fertilization models.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) mediates stimulus–secretion coupling in many cellular systems, including the ZP3-activated pathway in sperm. ZP glycoproteins produce sustained elevatations of sperm $[Ca^{2+}]_i$, as reported by ion-selective fluorescent probes (4, 14, 21, 25, 38). Several lines of evidence indicate that ZP signals promote Ca^{2+} influx through voltage-sensitive channels during initiation of acrosome reactions: *a*) ZP-dependent acrosome reactions and $[Ca^{2+}]_i$ elevations are inhibited by several different structural classes of voltage-sensitive Ca^{2+} channel antagonists, including 1,4-dihydropyridines (21, 25). *b*) Depolarization of sperm membrane potential with $[K^+]_o$ or with gramacidin D produces $[Ca^{2+}]_i$ elevations as well as acrosome reactions in the absence of ZP3. *c*) Depolarization-dependent responses are also attenuated by antagonists of voltage-sensitive Ca^{2+} channels (2, 25). This channel mediates an essential component of the ZP-dependent $[Ca^{2+}]_i$ elevation leading to acrosome reactions and is a likely site of action for the reported human contraceptive effects of 1,4-dihydropyridines (6, 31).

Present understanding of the mechanism of ZP3 signal transduction includes the identification of candidate sperm surface receptors (12, 39, 42) and the demonstration that both G protein (19, 27, 62) and tyrosine kinase (40, 57) signaling pathways are stimulated. Yet the coupling between receptor activation and Ca^{2+} channel opening is presently not well understood. In somatic cells, extracellular signals activate voltage-sensitive Ca^{2+} channels by depolarizing membrane potential as well as by modulating channel function through phosphorylation of channel proteins or by a rapid, membrane-delimited mechanism that may represent a direct interaction with G protein subunits (reviewed by 32 and 66).

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1. *Abbreviations used in this paper*: BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; $[Ca^{2+}]_i$, intracellular Ca^{2+}; $DiSBAC_2(3)$, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol; $DiSC_3(5)$, 3,3′-dipropylthiodicarbocyanine iodide; pH_i, internal pH; PTx, pertussis toxin; ZP, zona pellucida, mZP and bZP, mouse and bovine zona pellucida.

Here, the role of sperm membrane potential as an effector of ZP3 signals was examined using potentiometric fluorescent indicators. We find that ZP and ZP3 depolarize mouse and bovine sperm membrane potential by activating a pertussis toxin (PTx)–insensitive pathway that has the characteristics of a poorly selective cation channel. ZP signals also activate a second PTx-sensitive pathway that produces transient elevations of sperm internal pH (pH_i). Voltage-sensitive Ca^{2+} channels function as a coincidence detector by integrating these two ZP-derived signals. These results suggest that sperm membrane potential is an important effector of egg-induced sperm activation.

Materials and Methods

Solutions and Chemicals

Bovine and mouse sperm were incubated in dTALP and in Hepes-buffered CM media, respectively (68). Medium HNKG contains (mM) 25 Hepes, 120 Na^+-gluconate, and 5 K^+-gluconate. Medium NT contains (mM) 105 *N*-methyl-D-glucamine$^+$ ($NMDG^+$, adjusted to Cl^- form with HCl) and 22.6 Tris-carbonate, as well as concentrations of KCl, $CaCl_2$, $MgCl_2$, KH_2PO_4, Hepes, and metabolic substrates appropriate for bovine and mouse sperm (68). Additional modifications of these media, described under Results, are obtained by compensatory adjustment of [NaCl] or of [$NMDG^+Cl^-$]. PO_4^{2-} was removed during experiments with Cd^{2+}, Co^{2+}, and La^{3+}. Polyvinylpyrrolidone (M_r 40,000) was substituted for albumin in protein-free media. Free divalent metal ion concentrations were calculated using the ALEX program (1). All media also contained 22.5 mM lactic acid and 1 mM pyruvic acid and were adjusted to pH 7.4.

Chemicals were obtained from the following sources: bis-(1,3-diethylthiobarbituric acid) trimethine oxonol ($DiSBAC_2[3]$), 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3[5]$), ionomycin, and valinomycin from Molecular Probes, Inc. (Eugene OR); and pertussis toxin (PTx, with lots screened for activity towards intact sperm as described, 25), gramicidin D, and all other chemicals from Sigma Immunochemicals (St. Louis, MO).

Biological Preparations

Sperm were isolated from bovine seminal secretions and from mouse caudae epididymides, capacitated in vitro, and assayed for the ability to undergo ZP-initiated acrosome reactions and to fertilize eggs in vitro as described previously (25). Mouse and bovine ZPs were obtained from ovarian homogenates and soluble extracts were obtained as described (25). ZP glycoproteins were purified from mouse by SDS-PAGE under nonreducing conditions (8) and from bovine by two-dimensional PAGE. SDS and other electrophoretic reagents were removed by sequential dialysis in 8 M urea and in a 25 mM Hepes/125 mM NaCl, pH 7.4, medium (8). Acrosome reactions were assayed by the Coomassie blue dye–binding method (43, 45) and, in fluorescence microscopic experiments, by differential interference contrast microscopy (11).

Experiments determining the zona pellucida–dependent alteration of $Ca^{2+}{}_i$, pH_i, and acrosomal secretion used 100 μg/ml zona pellucida glycoprotein concentration, similar to dose–response relationships described previously (10, 23). In contrast, membrane depolarization occurs at ≤ 1 μg/ml zona pellucida glycoprotein (see Fig. 2). Such differences may reflect distinct experimental conditions, such as the removal of media protein before membrane potential determinations, as well as the characteristics of a bifurcated signal transduction mechanism (see Discussion).

Membrane Potential Determinations

Sperm population membrane potentials were determined from the fluorescent emission of $DiSBAC_2(3)$ and of $DiSC_3(5)$, as previously (68). Sperm were capacitated, resuspended in albumin-free media ($2.5–5 \times 10^6$ sperm/ml), incubated with fluorescent probes ($DiSBAC_2[3]$, 0.5 μM; $DiSC_3[5]$, 0.25 μM), and fluorescence emission monitored with an AlphaScan II spectrofluorometer, as described (68). Excitation/emission wavelength pairs of 530/575 nm and 620/670 nm (3 nm bandpass) were used for $DiSBAC_2(3)$ and $DiSC_3(5)$, respectively. Populations are stirred during data acquisition, particularly since some treatments (e.g., Cd^{2+} and Ni^{2+} experiments) reversibly inhibit sperm motility. Minimally disruptive conditions for stirring sperm suspensions were described (68). Values represent population-averaged, apparent membrane potentials.

pH_i and $[Ca^{2+}]_i$ Determinations

Sperm pH_i and $[Ca^{2+}]_i$ were calculated from the fluorescence emission of intracellular 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and fura 2, respectively. Methods for incorporation of dyes into sperm by incubation with membrane-permeant ester precursor forms, for sperm immobilization on Cell-Tak–coated coverslips, for image acquisition and analysis, and for the computation of apparent pH_i and $[Ca^{2+}]_i$ values were described previously (21, 24, 69).

Results

Effects of Zonae Pellucidae on Sperm Membrane Potential

The membrane potentials of capacitated mouse and bovine sperm populations were determined from the fluorescence emission of the anionic oxonol, $DiSBAC_2(3)$, and confirmed in parallel experiments with the cationic carbocyanine, $DiSC_3(5)$. Following correction for the contributions of mitochondrial potentials to probe signals, the membrane potentials of mouse sperm populations reported by these two probes were -54 ± 4 and -61 ± 7 mV, respectively, while values for bovine sperm were -51 ± 3 and -57 ± 8 mV, respectively. These values are similar to those obtained previously (25, 68) and are stable during several minutes in protein-free media (Fig. 1 *A* and *B*; before protein addition).

Solubilized ZPs depolarize mouse and bovine sperm membrane potentials. Fig. 1 *A* shows the effects of bovine ZPs (bZP, 100 μg/ml; 1 bZP ~27 ng protein; 23) on the membrane potential of homologous sperm as reported by $DiSBAC_2(3)$. In this experiment, the initial depolarization rate was 14.5 mV/min (Fig. 1 *A*, *dashed line*). Initial rates in both species are dose-dependent, as shown in Fig. 2. Mouse ZPs (mZP, 4 ng protein/mZP; 9) produce a half-maximal response at 44 ± 9 ng/ml and a maximal rate of 19.7 ± 3.7 mV/min at ≥250 ng/ml (Fig. 2 *A*), while bovine sperm exhibit a half-maximal response with 83 ± 19 ng/ml bZP and a maximal response of 17.8 ± 2.9 mV/min at ≥1 μg/ml (Fig. 2 *B*). Similar conclusions were drawn from parallel experiments in both species using the $DiSC_3(5)$ probe.

The specificity of this response is indicated by several control experiments. First, comparable concentrations of an unrelated glycoprotein (fetuin) produced only minor effects on membrane potential (<0.7 mV/min) that probably reflect direct binding of extracellular protein to probe. Similar effects were observed in sperm-free solutions. Second, depolarization occurred only when sperm were treated with homologous ZP solutions. Fig. 1 *B* shows that mZPs failed to depolarize bovine sperm membrane potential, and similar species selectivity was found when mouse sperm were treated with bZP. Finally, ZP-dependent responses require a polarized membrane potential in sperm. When membrane potentials were dissipated with gramicidin D, the subsequent addition of ZPs had only minor effects on probe fluorescence. These effects were similar to those observed in intact sperm treated with heterologous ZPs (not shown).

ZP-dependent depolarization precedes acrosome reac-

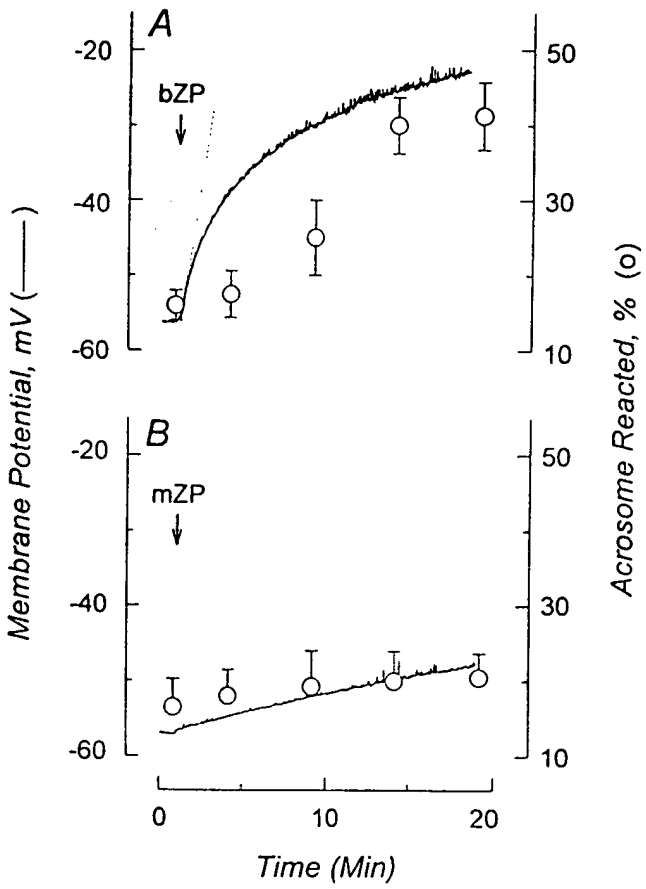

Figure 1. Effects of homologous and heterologous zonae pellucidae on the membrane potential and acrosome reaction of bovine sperm populations. Membrane potentials (—) were calculated from $DiSBAC_2(3)$ fluorescence emission and the occurrence of acrosome reactions (O) were determined as described in Materials and Methods. Shown are the calculated membrane potentials and incidence of acrosome reactions in bovine sperm populations before and after addition of: (*A*) soluble preparations of bovine zonae pellucidae (100 μg/ml; *arrow*), which produces a maximal dye response (see Fig. 2); and (*B*) soluble preparations of mouse zonae pellucidae (100 μg/ml; *arrow*). Fluorescence emission acquisition rate was 1 Hz and the initial rate of depolarization was estimated from linear fit of data obtained in the first 30 s (*A*, *dashed line*) following zona pellucida addition. Initial rates in the experiments shown here were (*A*) 14.5 mV/min, and (*B*) 0.4 mV/min. Acrosome reactions data represent the mean (± SD) of triplicate slides obtained from a sample at the indicated time point, with ~200 sperm assayed per slide.

tions in both species. The time courses of these two processes in bovine sperm populations are shown in Fig. 1 *A*. Such fluorescent responses do not reflect dye loss during the acrosome reaction since the $DiSBAC_2(3)$ anion and the $DiSC_3(5)$ cation both report ZP-dependent depolarizations, yet they redistribute in opposite directions. Other proteins (BSA, fetuin, and heterologous ZPs; Fig. 1 *B*) that fail to initiate acrosome reactions also do not produce apparent depolarization. These experiments are consistent with a model in which an early component of the ZP agonist–initiated signaling cascade includes depolarization of sperm membrane potential.

Effects of ZP3 on Sperm Membrane Depolarization

ZP3 binds specifically to sperm and initiates acrosome reactions, while other ZP glycoproteins do not participate in these processes (10, 45, 47). We examined the effects of highly enriched preparations of ZP glycoproteins on mouse and bovine sperm to determine whether depolarization is a specific effect of ZP3.

Fig. 2 *A* shows that mouse sperm are depolarized by mZP3 in a concentration-dependent manner. The half-maximal and maximally effective concentrations were 1.4 ± 1.1 and ~25 ng/ml. The initial depolarization rate at saturating concentrations of mZP3 was 14.8 ± 3.0 mV/min (background corrected) and accounts for essentially all of the membrane-depolarizing activity found in unfractionated mZPs. In contrast, mZP1 and mZP2 produced only small alterations (<4 mV/min) in membrane potentials. These alterations were not significantly different from those observed with fetuin and, again, are likely the results of direct interactions between protein and reporter dye.

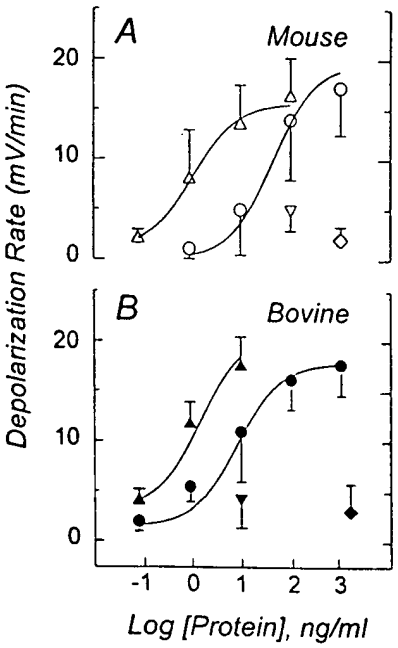

Figure 2. Initial rate of sperm membrane depolarization. Membrane potential was calculated from $DiSBAC_2(3)$ fluorescence emission and used to determine initial depolarization rates following addition of unfractionated zonae pellucidae, enriched fractions of zona pellucida glycoproteins, and control proteins to mouse (*A*, *open symbols*) and bovine sperm (*B*, *closed symbols*). Initial rates were determined as indicated in Fig. 1, and data represent the mean (± SD) of three to seven separate determinations. The effects of unfractionated zonae pellucidae and ZP3-enriched fractions were fit to the relationship: $R_C = [(R_{Max} + C)/(K + C)] + N$, where R_C and R_{max} are initial rates at each concentration of zona pellucida glycoproteins and the maximal initial rate, respectively; C is the concentration of zona pellucida glycoprotein; K is the apparent equilibrium constant for this reaction; and N is the nonspecific background depolarization rate following buffer addition. Values of R_{Max} (mV/min), K (ng/ml), and N for fit curves (—) were: mouse ZP, 19.7, 44, and 0.03; mouse ZP3, 14.8, 1.2, and 0.9; bovine ZP, 16.7, 83, 3.4; bovine class III glycoproteins, 21.1, 1.1, 1.4. Symbols: total ZP, (O, ●); ZP3-enriched fractions, (Δ, ▲); ZP1 + ZP2, (∇, ▼); fetuin, (◇, ◆).

Unlike the case in the mouse, bZP3 has not previously been identified. Fig. 3 shows that the bZP consists of three distinct classes of glycoproteins (Fig. 3, inset). To identify the group containing bZP3, individual groups were eluted from gel slices, dialyzed, and assayed for the ability to initiate acrosome reactions in bovine sperm. Fig. 3 shows that class III glycoproteins exclusively account for induction of acrosome reactions, whereas classes I + II lack this activity. Therefore, class III is enriched in bZP3.

Class III glycoproteins also account for the ability of bovine ZPs to depolarize sperm membrane, as shown in Fig. 2 *B* (half-maximal response ~1.1 ± 0.4 ng/ml; maximal response ~21.1 ± 3.1 mV/min). In contrast, classes I + II lack apparent stimulatory activity. These results demonstrate that in two mammalian species, fractions enriched in ZP3 depolarize sperm membrane potentials.

Permeation Characteristics of a Zona Pellucida-activated Depolarization Pathway

In order to deduce the ion selectivity of a ZP3-activated depolarization mechanism, we examined the effects of medium ion composition on the initial rates of response. Capacitated sperm were suspended in test media containing $DiSBAC_2(3)$. After fluorescence signals stabilized, ZP agonists were added (mZP, 250 ng/ml; bZP, 1 μg/ml) and the initial response rates were estimated from time courses similar to that in Fig. 1 *A*.

Fig. 4 illustrates the results obtained in a complete gamete culture medium. In this series of experiments, the initial depolarization rate following buffer addition was <1.5 mV/min (mouse, 1.4 ± 1.1 mV/min; bovine, 0.7 ± 0.5 mV/min) and was enhanced by 1.5–2-fold following addition of heterologous ZPs (Fig. 4) or fetuin (not shown). In contrast, homologous ZPs stimulated the initial rate by an additional 8–15-fold relative to control glycoproteins (mouse, 19.7 ± 3.7 mV/min; bovine, 17.8 ± 2.9 mV/min). In complete culture media, the ZP-dependent depolarization was not substantially reduced by either chelation of Ca^{2+} or of all divalent cations but was inhibited by 10 mM Cd^{2+} (not shown) or by 0.1 mM La^{3+} (Fig. 4). Cd^{2+} also inhibits the voltage-sensitive Ca^{2+} channel, but at 10–100-fold lower concentration (21), thereby differentiating that pathway from the depolarization mechanism described here. In the following series of experiments, La^{3+} and Cd^{2+} sensitivity provides a signature for the ZP3-activated pathway.

Anion composition did not significantly influence ZP-

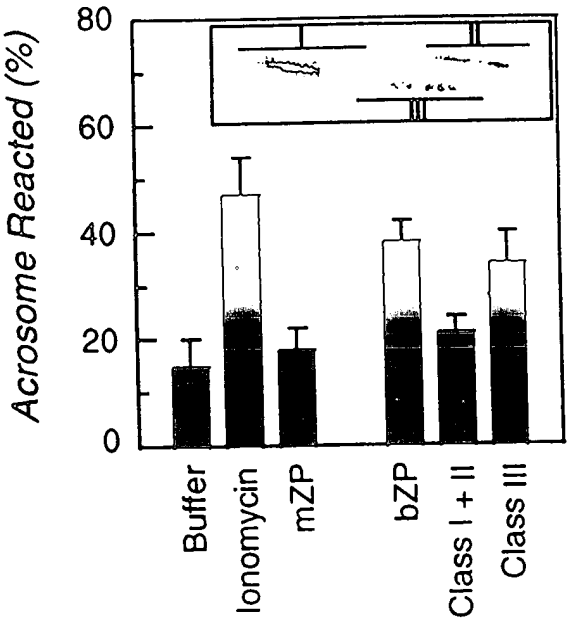

Figure 3. Induction of bovine sperm acrosome reactions by homologous zonae pellucidae. Capacitated bovine sperm were treated for 30 min with test reagents and then assayed for acrosome reactions. Treatment conditions were based on the concentrations of unfractionated bovine zona pellucida glycoproteins shown previously to produce maximal rates of acrosome reactions (see 24) and include: buffer, ionomycin (5 μM), unfractionated mouse zona pellucida extracts (mZP, 100 μg/ml), unfractionated bovine zona pellucida extracts (bZP, 100 μg/ml), class I + II bovine zona pellucida glycoproteins (data pooled from separate treatments; 10 μg/ml of each class), and class III bovine zona pellucida glycoproteins (10 μg/ml). Data represent the mean (± standard deviation) of three to five separate experiments with each experiment consisting of triplicate samples and with 100–200 sperm assayed per sample. (*Inset*) Autoradiograph of ^{125}I-labeled bovine zonae pellucidae following resolution by two-dimensional gel electrophoresis. Derived M_r and pI values are: Class I, 81–94 kD and 4.4–4.8; Class II, 94–112 kD and 7.0–8.4; and Class III, 70–76 kD and 5.7–7.3.

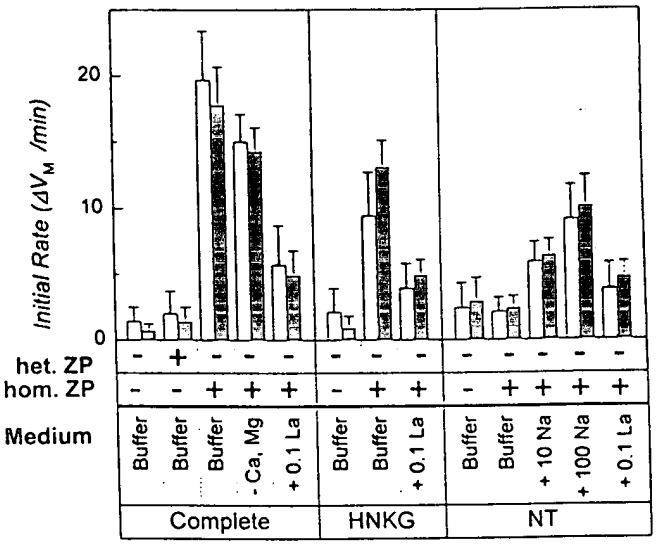

Figure 4. Ion selectivity of the zona pellucida–dependent depolarization mechanism. Initial depolarization rates for mouse (*filled bars*) and bovine sperm (*open bars*) were determined following treatment with solubilized zonae pellucidae (mouse, 250 ng/ml; bovine, 1 μg/ml; see Fig. 2). Zona pellucida glycoprotein concentrations promote maximal depolarization rates in complete culture media, as shown in Fig. 2. Sperm are treated with heterologous or homologous zonae pellucidae (*het. ZP* and *hom. ZP*). The composition of the base medium is indicated and described in detail in Materials and Methods. Complete media for bovine and mouse sperm were dTALP and Hepes-buffered CM, respectively. Modifications of these basic media are also indicated. Results represent the mean (± SD) of two to seven separate experiments.

evoked depolarizations, as shown in experiments in HNKG medium (Fig. 4). Robust responses occur following ZP treatment in a Na^+/K^+-based media containing either monovalent- or divalent anions (gluconate, Fig. 4; aspartate$^-$, Br^-, Cl^-, SCN^-, and SO_4^{2-}, data not shown). La^{3+} (Fig. 4) and Cd^{2+} (data not shown) inhibited responses in this simplified medium with potencies similiar to those observed in complete medium.

In contrast, the ZP-induced sperm membrane depolarization was dependent on the cation composition of the medium. Responses were reduced by >90% in medium NT (Fig. 4), where Na^+ and K^+ are replaced by the larger cation, *N*-methyl-D-glucamine$^+$ ($NMDG^+$). Subsequent substitution of $NMDG^+$ in NT medium with smaller mono- and divalent cations, such as Na^+ (Fig. 4), K^+, Ba^{2+}, Ca^{2+}, Mn^{2+}, or Ni^{2+} (not shown), restored ZP-dependent depolarization. The responses that are restored following Na^+ or Ca^{2+} readdition are inhibited by 0.1 mM La^{3+} and by 10 mM Cd^{2+}, reflecting the activation of that same depolarization mechanism as was observed in complete medium (Na^+, Fig. 4; Ca^{2+}, data not shown). Alternatively, ZP-evoked depolarizations could not be restored to sperm in medium NT by alterations of anion composition, as shown when Cl^- was replaced with other aspartate$^-$, Br^-, SCN^-, or SO_4^{-2}. Comparable results were obtained in a less extensive series of experiments with the bovine sperm using $DiSC_3(5)$. These observations suggest that ZP-dependent depolarization activates a mechanism with the anticipated characteristics of a poorly selective cation channel.

Role of Membrane Depolarization in Zona Pellucida–controlled Acrosome Reactions

Previous studies showed that pertussis toxin (PTx) inhibits an early component of ZP3 signal transduction and mitigates the induced elevations of $[Ca^{2+}]_i$ and pH_i (4, 21, 24) as well as the ZP-dependent acrosome reaction (19, 20). These effects most likely reflect the activation of sperm G_{i1} and/or G_{i2} by ZP3 (62). Yet PTx treatment of mouse and bovine sperm had only minor effects on ZP-induced depolarization. Treatment with 100 ng/ml PTx, which inhibits the induced acrosome reaction by >80% (19, 24), reduced the ZP-dependent initial depolarization rate of mouse sperm from 15.9 ± 2.0 to 13.4 ± 2.7 mV/min and of bovine sperm from 14.3 ± 2.6 to 11.1 ± 1.8 mV/min (n = 3, nonspecific background subtracted). We examined the coupling between membrane depolarization and Ca^{2+} channel activation in a series of single cell experiments. Stereotypic cells are illustrated in Fig. 5 and the results of these experiments are summarized in Fig. 6.

Fig. 5 *A* shows an example of a bovine sperm labeled with the fura 2 and BCECF fluorescent probes, permitting simultaneous determination of relative $[Ca^{2+}]_i$ and pH_i levels, respectively. Addition of bZPs produces a sustained $[Ca^{2+}]_i$ elevation, as reported previously (21, 24), as well as an increase in pH_i. This cell completes the acrosome reaction within 12–13 min (Fig. 5 *A*, *dashed line*). As shown in Fig. 6, addition of homologous ZPs elevated $[Ca^{2+}]_i$ by three- to fourfold and also increased the fraction of acrosome reacted sperm by threefold, relative to buffer-treated controls.

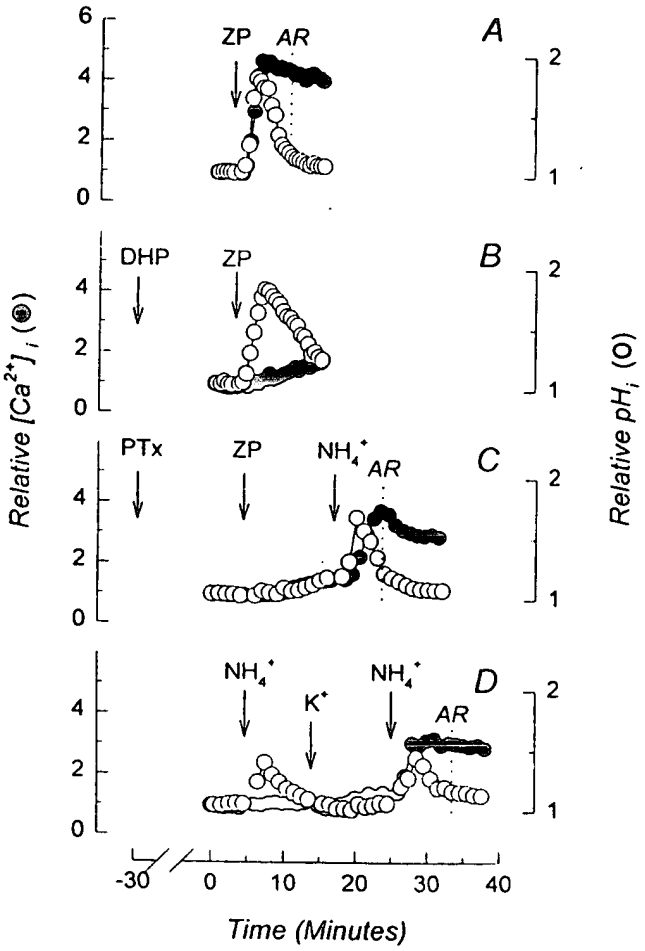

Figure 5. Bovine sperm pH_i modulates zona pellucida–dependent $[Ca^{2+}]_i$ responses. Relative $[Ca^{2+}]_i$ and pH_i were monitored simultaneously in cells doubly labeled with fura 2 (●) and BCECF (○), respectively. Resting $[Ca^{2+}]_i$ and pH_i levels are stable before treatment. (*A*) Addition of bovine zona pellucida extracts (ZP, 100 μg/ml) produces a transient alkalinization and a sustained elevation of $[Ca^{2+}]_i$. The acrosome reaction, detected using Normarski optics, occurred in this cell at 12–13 min (*AR*, *dashed line*). (*B*) Pretreatment (at t = −30 min) of sperm with 10 μM PN200-110 inhibits the ZP-induced elevation of $[Ca^{2+}]_i$ but had no effect on the induced elevation of pH_i. (*C*) Sperm are treated sequentially with 100 ng/ml pertussis toxin (at t = −30 min), with 100 μg/ml bZP (at t = 5 min), and with 10 mM NH_4^+ (at t = 17.5 min). The acrosome reaction occurred in this sperm at 23–24 min. (*D*) Treatment of sperm with 10 mM NH_4^+ (t = 5 min) in the absence of ZP produces transient alkalinization with little or no effect on $[Ca^{2+}]_i$ (see Fig. 6). K^+ depolarization of sperm membrane potential (80 mM $[K^+]_o$, substituted for Na^+) at t = 13 min produced monotonic increases in $[Ca^{2+}]_i$ and did not significantly alter pH_i. Addition of 10 mM NH_4^+ (t = 25 min) to sperm in K^+-based medium produces a transient alkalinization, sustained $[Ca^{2+}]_i$ elevation, and acrosome reaction (33–35 min).

As shown in Fig. 5 *B*, the ZP-dependent $[Ca^{2+}]_i$ elevation is inhibited by PN200-110 (21), the 1,4-dihydropyridine antagonist of some classes of voltage-sensitive Ca^{2+} channels. In these experiments, 10 μM PN200-110 reduced the peak $[Ca^{2+}]_i$ response to ZP stimuli from a 3.15 ± 0.13-fold increase to a 0.69 ± 0.15-fold elevation (n = 78 and 61

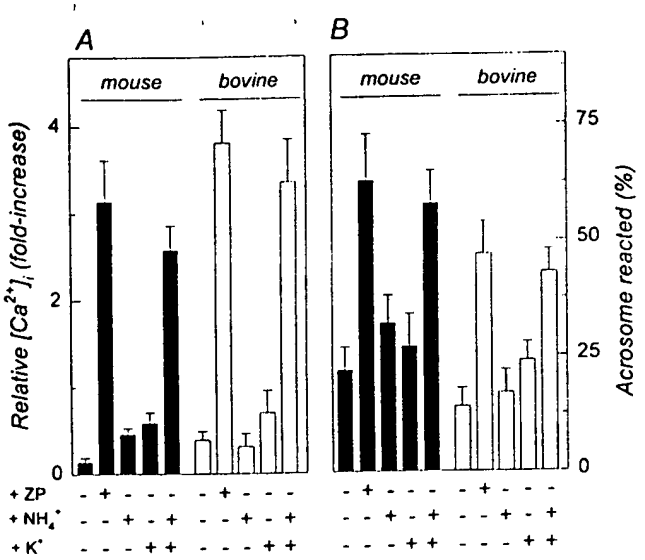

Figure 6. Sperm $[Ca^{2+}]_i$ is regulated by concerted membrane depolarization and internal alkalinization. Bovine (*open bars*) and mouse (*filled bars*) sperm were treated with buffer, with homologous zonae pellucidae (100 μg/ml), with 10 mM NH_4^+, with 80 mM K^+, and with the NH_4^+ + K^+, as described in Materials and Methods. (*A*) $[Ca^{2+}]_i$ was determined 5 min after zona pellucida addition, based on time courses similar to those illustrated in Fig. 5 *A*. Data represents the mean (± SD) of 41–77 cells in each treatment group, collected in three to five separate experiments. (*B*) Acrosome reactions were assayed 20 min after addition of zonae pellucidae. Data represent the mean (± SD) of cells assayed in *A*.

cells, respectively). There was a corresponding inhibition of the ZP-induced acrosome reaction: 10 μM PN200-110 reduces the level of acrosome reactions following ZP treatment from 47% (37/78) to 18% (11/61), similar to results obtained previously (21, 25). These observations are consistent with the notion that ion influx through voltage-sensitive channels is an essential component of the $[Ca^{2+}]_i$ response to ZP stimuli (21, 25).

In contrast, PN200-110 had no significant effect on the intracellular alkalinization promoted by ZP stimulus (Fig. 5 *B*). Sperm pH_i values were quantified in BCECF-loaded cells. This probe reports resting pH_i values in mouse and bovine sperm that had been subjected to capacitating incubations of 6.72 ± 0.04 (147 cells) and 6.61 ± 0.05 (92 cells), respectively. Approximately half of these cells exhibited a ZP-dependent alkalinization: 58% (85/147) of mouse sperm and 47% (43/92) of bovine sperm responded, with peak alkalinizations of 0.17 ± 0.06 and 0.13 ± 0.03, respectively. Extensive heterogeneity was observed in the time course of pH_i alterations. Among the cells that display ZP-dependent alkalinizations, ~70% (mouse, 62/85; bovine, 30/43) exhibit a transient pH_i increase (Fig. 5 *A*) in which rates of alkalinization and reacidification vary extensively. Alkalinization in the remaining cells is either sustained, similar to the $[Ca^{2+}]_i$ response shown in Fig. 1 *A* (mouse, 14/85; bovine, 9/43), or the cells display a biphasic response consisting of an initial rapid alkalinization followed by a slower secondary alkalinization (mouse, 9/85; bovine, 4/43). Within the subpopulation of sperm that exhibit ZP-dependent pH_i response, >85% [illegible] complete the acrosome reaction within 20 min, whereas acrosome reactions occur in <10% of the cells where alkalinizations are not observed. Taken together with previous reports (24, 25), these observations suggest that elevations of pH_i are a component of ZP signaling.

Fig. 5 *C* confirms that pretreatment with PTx substantially inhibits both the $[Ca^{2+}]_i$ and pH_i responses to ZP treatment. Only 11% of PTx-treated bovine sperm (17/158) and 17% of mouse sperm (16/94) exhibited ZP-evoked pH_i or $[Ca^{2+}]_i$ elevations, and there was a corresponding attenuation of ZP-induced acrosome reactions. Permeant weak bases, such as 10 mM NH_4^+, produce transient pH_i alkalinization (3, 52, 69). Such experimental alkalinization has only minor effects on $[Ca^{2+}]_i$ and on acrosome reactions in the absence of ZP/ZP3 but is sufficient to restore these responses to PTx-blocked sperm (Fig. 5 *C*). These results suggest that activation of a pH_i regulatory mechanism is the minimal PTx-sensitive element of ZP signal transduction.

Yet alkalinization with NH_4^+ in the absence of ZP/ZP3 fails to produce robust $[Ca^{2+}]_i$ responses (Figs. 5 *D* and 6; ref. 2): slow, monotonic increases in $[Ca^{2+}]_i$ occur in most cells (mouse, 41/57; bovine, 33/48) and result in a <0.5-fold increase in $[Ca^{2+}]_i$ (mouse, 0.46 ± 0.07-fold; bovine, 0.31 ± 0.04-fold). This is in contrast to the greater than threefold increases in $[Ca^{2+}]_i$ produced by ZP (Figs. 5 *A* and 6; ref. 21, 24). In addition, NH_4^+ alkalinization did not promote acrosome reactions in most sperm, as described previously (25). These observations suggest that additional signaling elements are activated by ZP contact, are not due to alkalinization, and are PTx insensitive. Similarly, Figs. 5 *D* and 6 show that K^+-depolarization in the absence of pH_i alkalinization also did not promote either a sustained $[Ca^{2+}]_i$ response (2) or initiate acrosome reactions (25). In contrast, concerted depolarization and alkalinization (with K^+_o and NH_4^+, respectively) induce both $[Ca^{2+}]_i$ elevations and acrosomal secretion even in the absence of treatment with ZP (Figs. 5 *D* and 6).

Discussion

There are two central observations in this study. First, sperm membrane potential is depolarized during contact with the ZP as a result of ZP3-dependent activation of a pathway with the anticipated characteristics of a cation channel. Second, depolarization mediates a component of ZP3 signaling leading to $[Ca^{2+}]_i$ elevations and acrosome reactions.

Fluorescent probes report that capacitated sperm have a resting membrane potential of −50 to −60 mV and that ZP3 depolarizes this potential by ~30 mV. The application of both anionic and cationic probes in two species lends confidence that depolarization is a general aspect of ZP3 signal transduction. Depolarization is produced by activating a mechanism with the anticipated characteristics of a cation channel, and the selectivity of this pathway was determined from the effects of ion depletion on the initial rate of probe response. This approach permits qualitative discrimination between ionic species that support the operation of this pathway and species that are not permissive, thereby establishing cation dependence. The more

typical method of establishing ion selectivity from the current reversal potential (32) was not feasible with sperm, where cytoplasmic dialysis using the whole-cell patch clamp has not been reported successfully.

Previously, we used fura 2 fluorescence and fluorescence-quenching methods to describe a cation transport mechanism in the bovine sperm head that is activated by ZPs (21). Divalent cation transport through that pathway was demonstrated, strongly supporting the notion that it is a channel. That cation transporter shares several functional features with the depolarization mechanism described here, including: cation selectivity (both are permeable to Ca^{2+}, Mn^{2+}, and Ni^{2+}), anion rejection, inhibition by high concentrations of Cd^{2+}, and activation by ZP/ZP3 through a PTx-insensitive mechanism. These two experimental approaches most likely reveal a single ZP3-regulated mechanism in the sperm head. The relationship of this depolarization mechanism to the other cation-selective channels that have been described in mammalian (16, 26, 56, 65) and echinoderm sperm (29) must be established.

ZP3-evoked depolarization is slow compared to the time course of activation of ion channels. The protracted kinetics may be due to several factors. First, mammalian sperm response to ZPs is unsynchronized and heterogeneous. Extensive cell-to-cell variation is observed in the lag time that precedes ZP-induced responses. In addition, only a subpopulation (25–50%) of sperm complete the capacitation process and respond to ZPs, further adding to population heterogenity (23, 61). This functional heterogeneity dominates the population kinetics of ZP-dependent $[Ca^{2+}]_i$ and acrosome reaction responses (21, 24, 38). The redistribution-type probes used here provide spatially averaged membrane potentials for populations and thus do not directly reflect the time course of either membrane depolarization in individual cells or the activation kinetics of a cation channel. Single cell determinations of membrane potential are required to assess the contribution of population heterogeneity in these responses. Second, cation channel density has not been determined and may contribute to the protracted kinetics. Finally, certain other secretory systems also exhibit slow depolarizations that are a component of the signal transduction mechanism (for example, 44).

The most likely function of ZP3-dependent depolarization during sperm–egg interaction is to participate in the control of Ca^{2+} channels. A model for ZP3-activated $[Ca^{2+}]_i$ responses during mammalian fertilization can be proposed based on the results of these and other experiments (Fig. 7). A central feature of this model is the ZP3-dependent activation of a sperm cation channel which, in the presence of an inwardly negative membrane potential (present study, 25, 68), mediates a depolarizing current. Insensitivity to PTx inhibition is a signature of this portion of the signaling pathway. Plausible mechanisms of ZP3-depolarization coupling include the direct activation of a ligand-gated cation channel as well as the mediation of PTx-insensitive transduction elements. In this regard, sperm possess both a ZP3-stimulated tyrosine kinase activity (39, 40) as well as the PTx-insensitive G proteins, G_q and G_z (60).

It is known that ZP3 also activates sperm pH_i regulatory

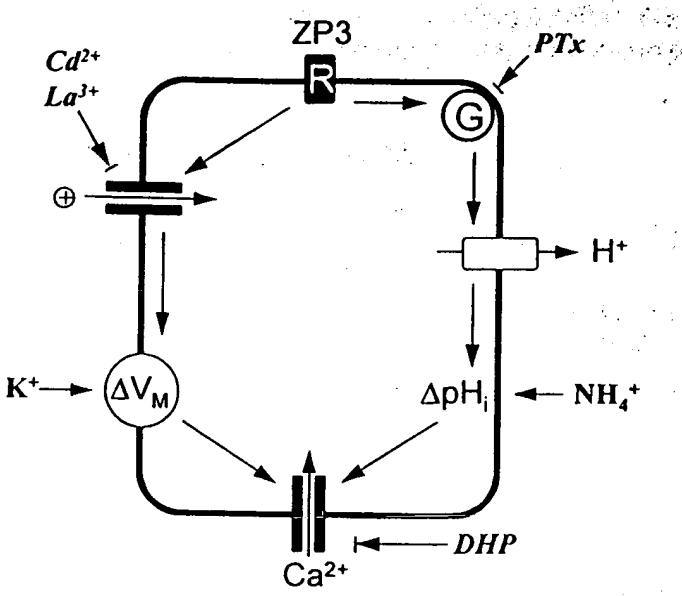

Figure 7. Model of ZP3 signal transduction. Binding of ZP3 to a complimentary receptor on sperm (*R*) initiates a bifurcated signaling pathway. *a*) A cation channel is activated through a pertussis toxin (PTx)-insensitive mechanism, leading to cation influx and membrane depolarization (ΔV_M). This limb is mimiced by high $[K^+]_o$ depolarization. *b*) A PTx-sensitive pathway is activated, leading to the stimulation of an acid efflux mechanism and to transient intracellular alkalinization (ΔpH_i). Transient alkalinization can be produced experimentally by application of NH_4^+ and other permeant weak bases (69). The inhibitory effects of PTx likely reflect the mediatory role of sperm G_{i1} and/or G_{i2}. These concerted responses regulate the activity of a voltage-sensitive, pH_i-modulated Ca^{2+} channel, leading to Ca^{2+} influx and triggering acrosome reactions. The figure also indicates the site at which signal transduction in inhibited by Cd^{2+}, La^{3+}, PTx, and dihydropyridines (*DHP*).

mechanisms (20, 24, 25, 38) and that the resultant alkalinization modulates membrane potential–dependent regulation of sperm Ca^{2+} channels and acrosome reactions (2, 25). Our data demonstrate that the induced alterations of pH_i constitute the PTx-sensitive step of ZP signal transduction. This is illustrated most directly by the effects of 10 mM NH_4^+. At this concentration, this permeant weak base does not induce $[Ca^{2+}]_i$ elevations (Figs. 5, *C* and *D* and 6) or acrosome reactions (Fig. 6; ref. 25) in the absence of ZP stimulation, yet it is sufficient to restore both the ionic and secretory response to ZPs in PTx-treated sperm (see Fig. 5 *C*). These observations strongly suggest that a pH_i-regulatory pathway is the major PTx-sensitive step in ZP3 signal transduction. Functional studies demonstrate that the principal regulators of sperm pH_i include a Na^+-dependent Cl^-/HCO_3^- exchanger and an anion-independent, arylaminobenzoate-sensitive regulator (69), while immunofluorescence and northern hybridization suggest that a Na^+-H^+ exchanger and a Na^+-independent Cl^-/HCO_3^- exchanger may also be present (48, 49). The pathway that mediates PTx-sensitive alkalinization in response to ZP3 stimuli has not been identified. In this regard, ZP3 activation of PTx-sensitive G_{i1} and G_{i2} has been demonstrated in detergent-permeabilized sperm (62) and may control a sperm pH_i-regulatory pathway.

Thus, these observations suggest that ZP3 activates a bifurcated signaling cascade consisting of the opening of a PTx-insensitive cation channel and the stimulation of a PTx-sensitive pH_i regulator (Fig. 7). Concerted depolarization and alkalinization are more effective in promoting $[Ca^{2+}]_i$ elevations than either signal alone. A branched signal transduction structure, with high- and low-affinity limbs, may account for the observed differences in potency of zona pellucida–dependent depolarizations and pH_i responses.

The $[Ca^{2+}]_i$ response mechanism functions operationally as the integrator of a coincidence detecting system. Similarly, voltage-sensitive Ca^{2+} channels in somatic cells (18, 34, 36, 41, 51, 58; reviewed in 41), as well as other ligand- and voltage-regulated channels (for example, 7, 13, 15, 17, 28, 30, 37, 55; reviewed in 46), are also modulated by pH_i. Frequently, such Ca^{2+} channel modulation is observed at values that are more acidic than the typical range of somatic cell pH_i and may function as a health sensor, curtailing cellular activity during internal acidification. In contrast, pH_i in mammalian sperm is relatively acidic, as determined by fluorescent probes (2, 3, 24, 50, 53, 59, 69) and by ^{31}P-NMR (54). Under these conditions, this mechanism of Ca^{2+} channel modulation may function within the physiological range of pH_i values and operate as a behavioral switch.

Given that sperm have only a single secretory granule and that the fertilizing ability of this cell is compromised when acrosome reactions are either induced prematurely or inhibited (22), it is anticipated that secretion may be regulated stringently. Dual modulation by membrane potential and pH_i provides a means of suppressing Ca^{2+} entry until ZP contact. The utility of a channel-based coincidence detector is illustrated by considering the storage state of sperm within the cauda epididymides. The luminal fluid of that compartment has a Na^+:K^+ ratio of 1:2 (33), sufficient to depolarize sperm membrane potential (23, 68). The relatively acidic conditions within epididymidal plasma (pH <7; ref. 33) may limit Ca^{2+} entry through voltage-sensitive channels and may account for the low levels of spontaneous exocytosis during storage.

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